



# Efficacy of reducing sugar and phenol–sulfuric acid assays for analysis of soluble carbohydrates in feedstuffs



Mary Beth Hall<sup>\*,1</sup>

U. S. Dairy Forage Research Center, USDA-Agricultural Research Service, Madison, WI 53706, USA

## ARTICLE INFO

### Article history:

Received 29 September 2012

Received in revised form 19 June 2013

Accepted 22 June 2013

### Keywords:

Analysis

Feed composition

Fructan

Nonstructural carbohydrate

Sugars

## ABSTRACT

Reducing sugar (RSA) and phenol–sulfuric acid (PSA) assays are commonly used to analyze water-soluble carbohydrates. However, questions have arisen as to their accuracy for measurements of feedstuffs with diverse carbohydrate profiles. This study evaluated the efficacy of RSA and PSA as they would commonly be applied in feed analysis laboratories in measuring a variety of purified carbohydrates. Carbohydrates analyzed were glucose (Glc), fructose (Fru), galactose (Gal), sucrose (Suc), maltose (Mal), lactose (Lac), raffinose (Raf), and inulin (Inu). Variations on the methods used were PSA using Suc (PSA-Suc) or Glc (PSA-Glc) as standard sugars, and RSA with a 50:50 Glc:Fru blend as the standard with four hydrolysis methods: acid hydrolysis with 0.037 M sulfuric acid (RSA-H<sub>2</sub>SO<sub>4</sub>) or 0.5 M hydrochloric acid (RSA-HCl), or enzymatic hydrolysis with invertase (RSA-Inv) or an enzyme blend including sucrase,  $\alpha$ -glucosidase, and  $\beta$ -galactosidase (RSA-EnzBl). Recovery of carbohydrate was calculated on a dry matter (DM) basis as (carbohydrate detected g/kg DM)/(carbohydrate present g/kg DM), with 'close to' complete recovery defined as values falling within the range of 920–1080 g/kg. Monosaccharide recovery did not differ between unhydrolyzed vs. hydrolyzed samples in RSA indicating no destruction of carbohydrate by hydrolysis method. For RSA, recoveries of Glc, Fru, and Gal were 979, 1042, and 706 g/kg, respectively. Such response differences among monosaccharides are inherent to RSA, and can affect carbohydrate recovery values. Methods that provided close to complete recovery by carbohydrate were: PSA-Suc and all RSA for Suc; PSA-Glc and RSA-EnzBl for Mal and Lac; PSA-Suc, RSA-H<sub>2</sub>SO<sub>4</sub>, RSA-HCl, and RSA-Inv for Raf; and RSA-H<sub>2</sub>SO<sub>4</sub> and RSA-HCl for Inu. None of the assays gave complete recovery of the diverse set of purified carbohydrates. Allowing a range of 920–1080 g/kg for recoveries on individual carbohydrates, RSA-H<sub>2</sub>SO<sub>4</sub> and RSA-HCl would give the closest to complete recovery values for feeds such as forage and soybean in which Suc, Raf, and Inu were important, whereas RSA-EnzBl would be useful in feeds such as forages or dairy products when Suc, Mal, and Lac are of interest. The allowed 920–1080 g/kg range of acceptable

**Abbreviations:** PSA, phenol–sulfuric acid assay; PSA-Glc, PSA with glucose as the standard sugar; PSA-Suc, PSA with sucrose as the standard sugar; RSA, reducing sugar assay; RSA-EnzBl, hydrolysis with an enzyme blend and RSA detection; RSA-H<sub>2</sub>SO<sub>4</sub>, sulfuric acid hydrolysis with RSA detection; RSA-HCl, hydrochloric acid hydrolysis with RSA detection; RSA-Inv, enzymatic hydrolysis with invertase and RSA detection; SED, standard error of the difference.

\* Correspondence address: U. S. Dairy Forage Research Center, USDA-ARS, 1925 Linden Drive, Madison, WI 53706, USA. Tel.: +1 608 890 0078; fax: +1 608 890 0076.

E-mail addresses: [marybeth.hall@ars.usda.gov](mailto:marybeth.hall@ars.usda.gov), [marybeth.hall@ars.usda.gov](mailto:marybeth.hall@ars.usda.gov), [mbhall@wisc.edu](mailto:mbhall@wisc.edu)

<sup>1</sup> Mention of any trademark or proprietary product in this paper does not constitute a guarantee or warranty of the product by the USDA or the Agricultural Research Service and does not imply its approval to the exclusion of other products that also may be suitable.

recoveries addresses the point that given very diverse carbohydrate complements of feeds, these assays will not be extremely precise, but may still be serviceable for diet formulation. The most accurate measurements will be achieved by selection of detection method, hydrolysis method, and carbohydrate standard to give greatest recovery of predominant carbohydrates in feedstuffs.

Published by Elsevier B.V.

## 1. Introduction

Reducing sugar and condensation assays are widely used for the analysis of water soluble carbohydrates in feeds both in research and commercial feed analysis laboratories. Methods such as HPLC that are capable of detecting specific carbohydrates have not been commonly used for commercial feed analysis due to cost and lower throughput of samples. Reducing sugar assays (RSA; e.g., method 974.06; [AOAC, 2005](#)) require hydrolysis of oligo- and polysaccharides to allow measurement of released monosaccharides. The most common hydrolysis conditions used are those designed to hydrolyze sucrose, which reflects its predominance in many plant-derived feedstuffs. Condensation methods such as the phenol-sulfuric acid assay (PSA; [DuBois et al., 1956](#)) do not require hydrolysis of the carbohydrates and detects mono-, oligo-, and polysaccharides. For any of these methods, it is recommended that the carbohydrate used to produce the standard curve be representative of the carbohydrates measured, because the colorimetric reactions are not stoichiometric and different carbohydrates give different absorbance responses. However, the directive regarding standard carbohydrate selection can be problematic when analyzing feedstuffs with diverse soluble carbohydrate compositions. Selection of a carbohydrate standard to use with detection of blends of carbohydrates will be imperfect, and selection of the correct blend of carbohydrates for the standard would typically be impractical, as it would require foreknowledge of carbohydrate composition and likely an individual standard for each feedstuff. Accordingly, selection of carbohydrates likely to predominate (e.g., sucrose, glucose, fructose) or glucose so that carbohydrates may be reported as “glucose equivalents” ([Brummer and Cui, 2005](#)) have been recommended.

In recent years, increased interest in addressing soluble carbohydrates in diet formulations for livestock and the concomitant increase in number of feed analyses run have raised questions regarding interpretation and accuracy of some of the values generated. Most commonly, the soluble carbohydrates have been analyzed by hydrolysis of a water extract and detection with RSA or 80% ethanol extracts measured with PSA ([Hall et al., 1999](#)). In particular, unexpectedly low values have been noted for feeds containing lactose, and low or negative estimates of long chain fructan content have been obtained when calculated as water-soluble minus ethanol-soluble carbohydrates analyzed as described above. Determination of the accuracy of methods as they are implemented in feed laboratories for measuring specific water-soluble carbohydrates will be useful to determine the utility of these analyses with different feedstuffs, and whether specific analytical approaches could improve accuracy.

The objective of this study was to evaluate factors that may affect recoveries of water soluble carbohydrates using RSA and PSA analyses. With a variety of purified water-soluble carbohydrates as the substrates, RSA with 4 different hydrolysis methods, and PSA with 2 different carbohydrate standards were evaluated.

## 2. Materials and methods

### 2.1. Samples

The purified carbohydrates used were glucose (Sigma G7021, purity 99.9%), fructose (Fluka 47739, purity 100%), galactose (Sigma 48259, purity 100%), maltose monohydrate (Sial M5885, purity 99.3%), sucrose (Fluka 84105, purity 100%), raffinose pentahydrate (Fluka 83400, purity 99.6%) (all purchased from Sigma-Aldrich Co., St. Louis, MO, USA), lactose monohydrate (Amend Drug & Chemical Co., Irvington, NJ, USA, purity 99%), and chicory inulin (a gift from P. Harrison, USDA-ARS, Forage and Range Research Laboratory, Logan, UT, USA). Sample solutions of 200 µg carbohydrate/mL were prepared in distilled water. Dry matter values for all samples were determined after drying overnight at 105 °C in a forced-air oven.

### 2.2. Hydrolysis methods for RSA

Two separate replicate analytical runs were performed for each of four hydrolysis methods tested. In each run, carbohydrate solutions were analyzed with addition of the specified acid or enzyme (hydrolyzed), or with addition of distilled water or buffer in place of the acid or enzyme (unhydrolyzed). The addition of buffer or water alone was included to assess the effect of run conditions on hydrolysis and recovery of samples separate from deliberate hydrolysis. Each run included reagent blanks with water as the sample and carbohydrate solutions (200 µg carbohydrate/mL). All samples within each treatment were run in duplicate within each analytical run; procedures below describe handling of individual replicates. Values of replicates within run were averaged to give a single value for each sample and treatment in each analytical run.

**H<sub>2</sub>SO<sub>4</sub> hydrolysis (RSA-H<sub>2</sub>SO<sub>4</sub>):** Acid hydrolysis with H<sub>2</sub>SO<sub>4</sub> was performed according to the method of [Bach Knudsen \(1997\)](#) modified to accommodate use of microfuge tubes. Three hundred microliters of 0.111 M H<sub>2</sub>SO<sub>4</sub> (prepared with 95–98% ACS reagent, #320501, Sigma-Aldrich Co., St. Louis, MO, USA) or distilled water were pipetted into separate 2 mL

microfuge tubes. Carbohydrate solution (600  $\mu$ L) was then pipetted into each microfuge tube. Tubes were immediately capped and vortexed. Samples were incubated in a circulating water bath at 80 °C for 70 min. Samples were then cooled to room temperature for 10 min in a basin of water at ambient temperature.

**HCl hydrolysis (RSA-HCl):** Acid hydrolysis with HCl was performed using a procedure developed for 96 place deep well reaction plates (P. Harrison, USDA-ARS, Forage and Range Research Laboratory, Logan, UT, USA, personal communication). For each sample, 50  $\mu$ L of 1.5 M HCl (prepared with ACS reagent, A144C-212, Fisher Scientific, Pittsburgh, PA, USA) or distilled water were pipetted into separate wells of a 96 well deep well reaction plate. Carbohydrate solution (100  $\mu$ L) was then pipetted into each well. The plate was covered with plastic adhesive film and incubated at 70 °C for 60 min in a recirculating water bath. Samples were then cooled to room temperature for 10 min in a basin of water at ambient temperature.

**Invertase hydrolysis (RSA-Inv):** Invertase (E-INVRT, Megazyme International Ireland, Bray, Co. Wicklow, Ireland, EC 3.2.1.26, 2000 U/mL) was diluted in 0.1 M acetic acid buffer (pH 4.6) to a concentration of 10 U invertase/mL. Carbohydrate solution (200  $\mu$ L) was pipetted into two 16 mm  $\times$  100 mm borosilicate tubes. To each tube 200  $\mu$ L of 0.1 M sodium acetate buffer (pH 4.6) was added or 200  $\mu$ L of diluted invertase solution. Tubes were covered with plastic film and incubated in a water bath at 50 °C for 20 min.

**Enzyme blend hydrolysis (RSA-EnzBI):** The enzyme preparation used (E-SUCRBG, Megazyme International Ireland, Bray, Co. Wicklow, Ireland) contained sucrose (E.C. 3.2.1.48, 28.3 U/mL),  $\alpha$ -glucosidase (E.C. 3.2.1.20, 433.3 U/mL), and  $\beta$ -galactosidase (E.C.3.2.1.23, 500 U/mL) in 5 mM sodium acetate buffer (pH 5.0). This enzyme mix is used for removal of sucrose, maltose, and lactose from dietary fiber determinations. Two hundred microliters of carbohydrate solution was pipetted into each of two 2 mL microfuge tubes. Fifty microliters of sodium acetate buffer (5 mM, pH 5.0) or enzyme solution was added to each tube. Tubes were vortexed to mix and incubated in a water bath at 40 °C for 60 min. Samples were centrifuged at 12,000  $\times$  g for 5 min before analysis of the supernatant.

### 2.3. Carbohydrate detection methods

A colorimetric RSA using *p*-hydroxybenzoic acid hydrazide (H9882, Sigma–Aldrich Co., St. Louis, MO, USA) (method 999.03, AOAC, 2005) was adapted to use 96 place deep well reaction plates and flat bottomed plates with absorbance read on a microplate reader spectrophotometer. Thirty microliters of distilled water, reagent blank, processed sample, or standard was pipetted into the deep well plate. Working reagent (500  $\mu$ L) was added to each well, the plate covered with plastic film, and incubated at 95 °C for 6 min. The plate was then cooled in a basin of water at ambient temperature for 10 min. Using a multichannel pipette, 200  $\mu$ L of solution for each replicate absorbance measure were transferred to individual wells in a flat bottom 96 well plate and read on a plate reader (ELx808 Absorbance Microplate Reader, BioTek US, Winooski, VT, USA) at an absorbance of 410 nm. A 50:50 blend of glucose:fructose at concentrations of 0, 75, 150, 200, and 275  $\mu$ g/mL was used for the standard solutions which were prepared with 0.2% benzoic acid as a preservative.

The colorimetric PSA was performed according to the method of DuBois et al. (1956) with samples vortexed after each liquid addition except the first. Two separate analytical runs were performed in which a solution of each of the purified carbohydrates (200  $\mu$ g carbohydrate/mL) were diluted 1 in 5 with distilled water and analyzed in triplicate. Water was used as the reagent blank. Separate sucrose and glucose standard solutions of 0, 33, 67, and 100  $\mu$ g/mL were prepared with 0.2% benzoic acid as a preservative. These two sets of standards were used to generate separate glucose (PSA-Glc) or sucrose (PSA-Suc) based standard curves with which to interpret sample absorbance values. The triplicate absorbance values for each carbohydrate were averaged to give a single, average absorbance value per sample. This approach gave a single measured carbohydrate value for each carbohydrate in each analytical run. Absorbances were read at 490 nm.

For both detection methods, average absorbance of reagent blanks within an analytical run were subtracted from sample absorbance values for calculations of detected carbohydrate relative to the standard curves. Previous investigations in our laboratory showed no effect on absorbance values of RSA and PSA of inclusion of 0.2% benzoic acid in standard solutions as compared to use of distilled water alone (data not shown). Recovery values for samples were calculated as (carbohydrate detected g/kg DM)/(carbohydrate present kg/kg DM). Recovery values were not adjusted for the weights of released monosaccharides as compared to the unhydrolyzed carbohydrate, as the degree of polymerization would be unknown for a blend of carbohydrates detected in feedstuffs. A value of 1000 g/kg was termed “complete recovery” relative to the amount of carbohydrate assayed.

### 2.4. Statistical analysis

The data sets analyzed were comprised of single values for each carbohydrate sample and each treatment (detection method, hydrolysis method) from each of two separate, replicated analytical runs, giving a total of 2 values per sample per treatment. Standard deviations describing the precision of the methods across analytical runs were calculated from the mean values for each substrate in each method determined in each run. All data were analyzed with carbohydrate recovery as the dependent variable. Several approaches were taken to data evaluation. Each carbohydrate was analyzed individually to assess the effect of hydrolysis method (the independent variable) on RSA results, with separate analyses performed for samples that were hydrolyzed and those that were carried through each run without hydrolysis. Comparisons of RSA recovery values for hydrolyzed, unhydrolyzed, and hydrolyzed minus unhydrolyzed values for the monosaccharides glucose, fructose, and galactose were made with carbohydrate, hydrolysis method, and carbohydrate by method as the independent

**Table 1**  
Reducing sugar assay recovery of carbohydrate DM g/kg.<sup>e,f</sup>

Substrate	H <sub>2</sub> SO <sub>4</sub>	HCl	Inv	EnzBl	SED	P
<i>Unhydrolyzed</i> <sup>g</sup>						
Glucose	966 (1)	959 (16)	928 (31)	972 (4)	18	0.20
Fructose	1031 (23)	1022 (39)	1051 (59)	1036 (6)	37	0.89
Galactose	701 (14)	682 (14)	701 (51)	708 (2)	27	0.78
Sucrose	18 (11)	14 (8)	11 (6)	13 (1)	7	0.84
Maltose	596 (40)	570 (23)	564 (9)	600 (6)	24	0.43
Lactose	429 (1)	431 (1)	416 (24)	452 (6)	13	0.21
Raffinose	23 (15)	41 (36)	12 (8)	26 (5)	20	0.59
Inulin	80 (5)	86 (18)	62 (4)	66 (2)	9	0.17
<i>Hydrolyzed</i> <sup>g</sup>						
Glucose	980 (6)	971 (28)	982 (16)	984 (14)	18	0.89
Fructose	1051 (37)	1024 (30)	1063 (42)	1030 (5)	32	0.63
Galactose	682 (3)	701 (14)	712 (31)	727 (6)	17	0.21
Sucrose	1036 (15)	1027 (33)	1040 (8)	1058 (25)	22	0.59
Maltose	614 (27) <sup>a</sup>	616 (24) <sup>a</sup>	587 (17) <sup>a</sup>	1080 (5) <sup>b</sup>	20	<0.01
Lactose	443 (1) <sup>a</sup>	460 (17) <sup>a</sup>	447 (16) <sup>a</sup>	939 (3) <sup>b</sup>	12	<0.01
Raffinose	942 (40) <sup>b</sup>	930 (33) <sup>b</sup>	924 (4) <sup>b</sup>	33 (0) <sup>a</sup>	26	<0.01
Inulin	1065 (64) <sup>b</sup>	1056 (50) <sup>b</sup>	256 (37) <sup>a</sup>	122 (1) <sup>a</sup>	45	<0.01

<sup>a,b,c,d</sup> Values in same line with different letters differ,  $P < 0.05$ .

<sup>e</sup> H<sub>2</sub>SO<sub>4</sub> = acid hydrolysis with sulfuric acid; HCl = acid hydrolysis with hydrochloric acid; Inv = enzymatic hydrolysis with invertase; EnzBl = enzymatic hydrolysis with an enzyme blend; SED = standard error of the difference.

<sup>f</sup> Values in parentheses are standard deviations.

<sup>g</sup> Unhydrolyzed = samples analyzed with water or buffer substituted for acid or enzyme and used to evaluate the effects of run conditions; Hydrolyzed = samples analyzed with acid or enzyme added.

variables. Comparison within carbohydrate across all hydrolysis and detection methods was performed with method as an independent variable. The standard deviation values calculated across runs for PSA and RSA methods were analyzed with a model that contained sample and method (PSA-standard and RSA-hydrolysis method). Mean separation was performed using the Tukey–Kramer test when a main effect had  $P < 0.05$ . All statistical analyses were performed using the MIXED procedure of SAS (2010). Data in tables are the arithmetic means and (standard deviation) calculated from values determined in two separate analytical runs.

### 3. Results

#### 3.1. Effect of RSA hydrolysis methods on recovery values

Recovery values for unhydrolyzed individual carbohydrates did not differ across hydrolysis protocols ( $P > 0.16$ ; Table 1). Glucose, fructose, galactose, and sucrose recoveries after hydrolysis also did not differ among methods ( $P > 0.20$ ). The RSA-EnzBl gave the greatest post-hydrolysis recoveries for maltose and lactose ( $P < 0.05$  for both). Hydrolysis with acids or RSA-Inv gave similar ( $P > 0.05$ ) recoveries for raffinose which were greater than when RSA-EnzBl was used ( $P < 0.05$ ). Hydrolysis of inulin with acids gave similar ( $P > 0.05$ ) recoveries that were greater ( $P < 0.05$ ) than the enzymatic methods which did not differ ( $P > 0.05$ ).

Recovery values differed among monosaccharides whether or not they were subject to hydrolysis. Recoveries of unhydrolyzed monosaccharides differed by monosaccharide (956, 1035, and 698 g/kg for glucose, fructose, and galactose, respectively; standard error of the difference (SED) = 14,  $P < 0.01$ ), but not by hydrolysis method ( $P = 0.73$ ) or by the interaction of hydrolysis method by monosaccharide type ( $P = 0.73$ ). Recovery values for glucose, fructose, and galactose subject to hydrolysis also differed from each other (979, 1042, and 706 g/kg, respectively, SED = 12,  $P < 0.01$ ) but did not differ by hydrolysis method used ( $P = 0.48$ ) or by the interaction of monosaccharide and method ( $P = 0.54$ ). The differences for hydrolyzed minus unhydrolyzed recovery values did not differ by monosaccharide ( $P = 0.23$ ), hydrolysis method ( $P = 0.32$ ), or their interaction (0.22) (SED = 10; data not shown).

#### 3.2. Effect of phenol–sulfuric and reducing sugar methods on recovery values

All carbohydrates tested, including monosaccharides, showed differences in recovery values among PSA and RSA methods ( $P < 0.01$  for all carbohydrates; Table 2). For glucose, PSA-Suc overestimated glucose recovery by 282 g/kg and differed from all other methods ( $P < 0.05$ ). The other methods gave glucose recoveries ranging from 971 to 1002 g/kg and did not differ from each other ( $P > 0.05$ ). Recovery values for fructose were greatest and closest to complete recovery for RSA methods (average 1034 g/kg, no difference among RSA methods;  $P < 0.05$ ), and substantially underestimated by both PSA analyses, which differed from each other and from the RSA methods ( $P < 0.05$ ). The value closest to complete recovery for galactose

**Table 2**Comparison of carbohydrate recovery values for phenol–sulfuric acid assay and reducing sugar assay methods.<sup>e,f</sup>

Substrate	PSA-Glc	PSA-Suc	RSA-H <sub>2</sub> SO <sub>4</sub>	RSA-HCl	RSA-Inv	RSA-EnzBl	SED	P
Glucose	1002 (3) <sup>a</sup>	1282 (12) <sup>b</sup>	980 (6) <sup>a</sup>	971 (28) <sup>a</sup>	982 (16) <sup>a</sup>	984 (14) <sup>a</sup>	15	<0.01
Fructose	496 (3) <sup>a</sup>	637 (4) <sup>b</sup>	1051 (37) <sup>c</sup>	1024 (30) <sup>c</sup>	1051 (59) <sup>c</sup>	1030 (5) <sup>c</sup>	26	<0.01
Galactose	767 (25) <sup>b</sup>	982 (27) <sup>c</sup>	682 (3) <sup>a</sup>	701 (14) <sup>ab</sup>	701 (51) <sup>ab</sup>	727 (6) <sup>ab</sup>	20	<0.01
Sucrose	783 (6) <sup>a</sup>	1003 (13) <sup>b</sup>	1036 (15) <sup>b</sup>	1027 (33) <sup>b</sup>	1040 (8) <sup>b</sup>	1058 (25) <sup>b</sup>	19	<0.01
Maltose	1051 (9) <sup>b</sup>	1345 (2) <sup>c</sup>	614 (27) <sup>a</sup>	616 (24) <sup>a</sup>	587 (17) <sup>a</sup>	1080 (5) <sup>b</sup>	52	<0.01
Lactose	948 (9) <sup>b</sup>	1214 (5) <sup>c</sup>	443 (1) <sup>a</sup>	460 (17) <sup>a</sup>	447 (16) <sup>a</sup>	939 (3) <sup>b</sup>	11	<0.01
Raffinose	815 (5) <sup>b</sup>	1044 (2) <sup>d</sup>	942 (40) <sup>c</sup>	930 (33) <sup>c</sup>	924 (4) <sup>c</sup>	33 (0) <sup>a</sup>	21	<0.01
Inulin	596 (6) <sup>b</sup>	765 (9) <sup>b</sup>	1065 (64) <sup>c</sup>	1056 (50) <sup>c</sup>	256 (37) <sup>a</sup>	122 (1) <sup>a</sup>	37	<0.01

<sup>a,b,c,d</sup> Values in same line with different letters differ,  $P < 0.05$ .<sup>e</sup> PSA = phenol–sulfuric acid assay; RSA = reducing sugar assay; Glc = glucose standard with PSA; Suc = sucrose standard for PSA; H<sub>2</sub>SO<sub>4</sub> = acid hydrolysis with sulfuric acid and RSA; HCl = acid hydrolysis with hydrochloric acid and RSA; Inv = enzymatic hydrolysis with invertase and RSA; EnzBl = enzymatic hydrolysis with an enzyme blend and RSA; SED = standard error of the difference.<sup>f</sup> Values in parentheses are standard deviations.

was achieved with PSA-Suc, which differed from all other methods ( $P < 0.05$ ). The average of all RSA methods underestimated galactose recovery by 297 g/kg.

Sucrose recovery did not differ among PSA-Suc and all RSA hydrolysis methods ( $P > 0.05$ ) giving values of 3–58 g/kg in excess of complete recovery; only PSA-Glc gave a substantial underestimate and differed from all other assays ( $P < 0.05$ ; Table 2). Values for maltose recovery were PSA-Suc > PSA-Glc, RSA-EnzBl > RSA-H<sub>2</sub>SO<sub>4</sub>, RSA-HCl, and RSA-Inv ( $P < 0.05$ ). Values closest to complete recovery for maltose were achieved by PSA-Glc and RSA-EnzBl. PSA-Suc overestimated maltose recovery by more than 300 g/kg, whereas RSA with other hydrolysis methods underestimated the amount by 386–413 g/kg. The ranking of lactose recoveries were PSA-Suc > PSA-Glc, RSA-EnzBl > RSA-H<sub>2</sub>SO<sub>4</sub>, RSA-HCl, and RSA-Inv ( $P < 0.05$ ). Lactose recovery was underestimated by an average of 550 g/kg by the RSA acid and invertase hydrolysis methods. The RSA-EnzBl and PSA-Glc gave values only 52–61 g/kg less than complete lactose recovery, whilst PSA-Suc overestimated by 214 g/kg. Raffinose recoveries were PSA-Suc > RSA-H<sub>2</sub>SO<sub>4</sub>, RSA-HCl, RSA-Inv > PSA-Glc > RSA-EnzBl ( $P < 0.05$ ). PSA-Suc gave slightly greater than complete recovery of raffinose by 44 g/kg, whereas RSA with acid or invertase hydrolyses were slightly low by 58–76 g/kg. PSA-Glc and RSA-EnzBl both gave substantially lower recoveries for raffinose, with the RSA-EnzBl value close to zero. Inulin recovery rankings were RSA-H<sub>2</sub>SO<sub>4</sub>, RS-HCl > PSA-Suc, PSA-Glc > RSA-Inv, and RSA-EnzBl. The closest to complete recoveries for inulin were for RSA with acid treatments. Both PSA methods and RSA-Inv or RSA-EnzBl markedly underestimated inulin.

The standard deviations of carbohydrate recoveries for the different methods across analytical runs did not differ among samples ( $P = 0.30$ ), but did differ among methods ( $P < 0.01$ ). Mean values for standard deviation by method were 8, 9, 24, 29, 21, and 7 g/kg for PSA-Glc, PSA-Suc, RSA-H<sub>2</sub>SO<sub>4</sub>, RSA-HCl, RSA-Inv, and RSA-EnzBl, respectively. The PSA and RSA-EnzBl did not differ from each other ( $P > 0.05$ ), and RSA with acids or invertase hydrolyses did not differ from each other ( $P > 0.05$ ), however, these two groupings differed from each other ( $P < 0.05$ ).

## 4. Discussion

### 4.1. RSA of monosaccharides

The RSA recovery values for monosaccharides are instructive for understanding the effects of the hydrolysis methods and of the RSA detection method itself. That unhydrolyzed and hydrolyzed recovery values did not differ from each other for glucose, fructose and galactose indicates that the hydrolysis methods and run conditions used did not destroy the monosaccharides. The acid hydrolysis methods used in this study were developed for hydrolysis of sucrose, and to avoid the destruction of released fructose which is more sensitive than glucose to acid conditions (Harding et al., 1936). These results allow some assurance that free and total released monosaccharides are available for detection with the hydrolysis methods tested.

The differences among monosaccharides in their recovery values are inherent to RSA. When hydrolyzed monosaccharide recovery values are divided by the recovery value of glucose to show responses relative to glucose, glucose = 1.00, fructose = 1.06, and galactose = 0.73 in the present study. These results are numerically similar to values reported in previous studies for this RSA (glucose = 1.00, fructose = 1.02, galactose = 0.78; Lever, 1973), for the Folin–Wu alkaline copper method (glucose = 1.00, fructose = 1.05, galactose = 0.75; Weinbach and Calvin, 1935), and for the Somogyi–Shaffer–Hartmann alkaline ferricyanide method (glucose = 1.00, fructose = 1.03, galactose = 0.70; Weinbach and Calvin, 1935). The monosaccharides gave the same pattern of response irrespective whether the studies used glucose (Weinbach and Calvin, 1935) or 50:50 glucose:fructose (present study) as the standard carbohydrate. That the monosaccharides differ in response in a RSA is problematic for achieving accurate measurement of blends of carbohydrates present in feedstuffs. Use of any single monosaccharide or simple combination of monosaccharides as the standard will over- or underestimate the amount of other monosaccharides present, unless the carbohydrates in the sample have the same profile of monosaccharides as the standard. Accordingly, the use of glucose as the standard so that carbohydrates may be reported as “glucose equivalents”



(Brummer and Cui, 2005) will not improve accuracy of feed composition measurement nor reliability of interpretation of the measures. The decision to use a 50:50 blend of glucose and fructose in the present study was based on the assumption that such a standard would be appropriate in most forage materials where sucrose or equal proportions of glucose, and fructose predominate. However, carbohydrate contents of feedstuffs such as soybean meal that contain galacto-oligosaccharides, or whey permeate which contains lactose will be underestimated due to the lesser response of galactose, even given complete hydrolysis of these carbohydrates. In these cases, more appropriate standard sugars would be recommended, and a more accurate detection method if available.

#### 4.2. RSA of oligo- and polysaccharides and efficacy of hydrolysis methods

The RSA are designed to detect reactive monosaccharides or sugar residues, irrespective of the relative response of the reacting monosaccharide. Theoretical values for complete recovery of carbohydrate on a monosaccharide basis are 1000 g/kg for monosaccharides, 1050 g/kg for disaccharides, 1070 g/kg for trisaccharides, and 1111 g/kg for inulin based on the weight of released monosaccharides divided by the weight of the unhydrolyzed substrate. Values less than these suggest destruction or incomplete hydrolysis, though greater or lesser recoveries can also be a function of the response of a blend of reactive monosaccharides in a sample relative to a carbohydrate standard that contains a different profile of monosaccharides. In the present study, only recovery as related to the mass of unhydrolyzed carbohydrate is considered because, as applied to feedstuffs, there will typically be no way to determine degree of polymerization, extent of hydrolysis, and monosaccharide profile all of which affect response.

Responses of unhydrolyzed oligo- and polysaccharide carbohydrates to RSA is dictated by the presence of a reactive or unreactive sugar residue at the reducing end of the molecules. The reactivity of the reducing end sugar is determined by whether it is bound to the carbohydrate so that the monosaccharide ring stays closed (unreactive) or can open (reactive). Sucrose, raffinose, and inulin have an unreactive glucose at the reducing end of the molecule, hence the small responses of the unhydrolyzed molecules. In contrast, both maltose and lactose have reactive glucose at the reducing end. However, the responses were greater than (maltose) or less than (lactose) a predicted response of 525 g/kg which would be half the recovery of a disaccharide that released two monosaccharides. Lever (1973) also showed that maltose and lactose gave greater and lesser responses, respectively, relative to glucose on an equimolar basis with the same RSA as used in the present study.

The acid hydrolysis methods tested were designed for and achieved good recovery with sucrose; recovery of inulin was approximately 6% greater than complete recovery. The acid treatments used were apparently ineffective for hydrolysis of maltose and lactose with hydrolyzed and unhydrolyzed values being numerically similar. Acid hydrolysis of raffinose gave greater than 90% recovery, but the incomplete recovery may have been due to the lesser response of galactose relative to the glucose + fructose standards. However, it is also likely that the acid conditions used released fructose, but did not completely hydrolyze the trisaccharide (Browne, 1912, p. 737). Overall, the acid hydrolysis methods allowed good recovery of sucrose and inulin, substantial recovery of raffinose, but only partial recovery of lactose and maltose.

The use of enzymes has the advantage of specificity so that particular carbohydrates can be targeted for analysis. Invertase gave comparable recoveries to the acid hydrolyses for both sucrose and raffinose, but had small effect on maltose, lactose, and inulin. The enzyme blend gave good recoveries on sucrose and maltose, and the greatest recovery for lactose of all the hydrolysis treatments tested. Similar to the situation with raffinose, the reduced response of galactose in the RSA would reduce the apparent recovery of lactose with glucose + fructose used as standards. Of the hydrolysis methods tested, the enzyme blend gave the most complete recovery of mono- and disaccharides though the recovery of lactose was lower than the other sugars.

#### 4.3. Comparison of PSA and RSA methods

There was no single method that obtained complete recoveries across all carbohydrates tested. This was not a function of repeatability of the assays, as PSA and RSA applied to individual carbohydrates gave good precision based on the standard deviation values of recoveries for analyses made in separate analytical runs. Not surprisingly, the PSA were most accurate when analyzing the carbohydrate that served as the standard used for the assay. In general, both PSA gave low recoveries to fructose and to inulin, a fructose polymer, as compared to the RSA which tended to slightly overestimate them. The overestimation by RSA was a function of the greater response of fructose relative to a glucose + fructose standard. The PSA gave close to complete recoveries for 3 carbohydrates each: PSA-Suc gave the closest recovery estimates (within  $1000 \pm 56$  g/kg) for galactose, sucrose, and raffinose, whereas PSA-Glc gave close estimates for glucose, maltose, and lactose. With very different recoveries among assays, it is not surprising that attempting to treat PSA and RSA results as additive in order to estimate fractions such as fructans by difference may give negative numbers and is not a reliable approach.

The RSA gave close to complete recovery values for up to 5 carbohydrates in a given hydrolysis method, but the specific carbohydrates included differed by method. The RSA that gave the numeric recoveries closest to 1000 g/kg for 5 carbohydrates were RSA-H<sub>2</sub>SO<sub>4</sub>, RSA-HCl, and RSA-EnzBI. For RSA-H<sub>2</sub>SO<sub>4</sub> and RSA-HCl glucose, fructose, sucrose, raffinose, and inulin showed recoveries in the range of 930–1065 g/kg. For RSA-EnzBI, glucose, fructose, sucrose, maltose and lactose gave recoveries of 939–1080 g/kg. Galactose containing carbohydrates gave the lowest recovery in both methods. All three assays

could similarly detect free glucose and fructose; substantial amounts of free galactose are not a common constituent of feedstuffs, so low recovery of this monosaccharide should not typically be a major issue.

If one was to choose among these assays, RSA with acid hydrolysis would give the closest to complete recovery values for feeds such as forage and soybean in which sucrose, raffinose, and inulin were important, whereas RSA-EnzBI would be useful in feeds exclusive of cool season grasses and galacto-oligosaccharide sources when sucrose, maltose, and lactose were of interest. With 50:50 glucose:fructose as the standard carbohydrate, recovery values for feeds in which sucrose predominates should be quite good with any of these three RSA methods (Table 2).

Allowing a range of 920–1080 g/kg as acceptable for recoveries on individual carbohydrates addresses the point that given the diverse array of water-soluble carbohydrates present in feeds, the assays will not be extremely precise, but likely are still sufficiently serviceable for feed analysis for diet formulation. The impact on diet formulation of the differing recoveries will depend on the water-soluble carbohydrate content and composition of the feedstuffs. Regarding the impact of content, such allowable recoveries will give maximum deviations in water-soluble carbohydrate content of 10 g/kg and 20 g/kg in feedstuffs containing 125 and 250 g/kg, respectively, of these carbohydrates. The RSA-EnzBI will still underestimate lactose content of dairy products where lactose or its constituent sugars should be the sole carbohydrates present. An evaluation of RSA-EnzBI or other enzymatic hydrolysis with galactosidase activity with use of a 50:50 galactose:glucose standard would be useful in such a case to obtain maximal recoveries.

Although it was not evaluated in this study, another consideration regarding use of RSA and PSA involves interfering substances that may be present in the extracts of feed samples. Noncarbohydrate interfering substances can inappropriately increase or decrease analytical values. They may not preclude use of a given assay if they can be removed prior to analysis, if results can be corrected for their impact, or if the effect is deemed to be small enough to be of minimal concern. Reducing sugar assays can be affected by protein, minerals, and reducing substances, such as naturally occurring antioxidants, but the effects vary by the chemistry of the RSA (Lever, 1973; Southgate, 1976). Interferences in the PSA include sodium azide (Buyse and Merckx, 1993), cellulosic lint and other insoluble carbohydrates, and  $\text{NO}_3^-$  and  $\text{Fe}^{3+}$  (found in soil extracts; Marten and Frankenberger, 1990).

## 5. Conclusions

Although commonly used as broad spectrum water-soluble carbohydrate analyses, neither the reducing sugar assay nor phenol-sulfuric acid assay as they are performed in feed analysis laboratories gave complete recovery of the diverse set of purified carbohydrates. Certain acid and enzymatic hydrolysis methods used with reducing sugar analysis show promise for analysis of feeds containing specific subsets of water-soluble carbohydrates; all reducing sugar methods gave good recoveries for glucose, fructose, and sucrose. Selection of standard sugars to more closely match the released monosaccharide profile of particular carbohydrates (e.g., sucrose vs. lactose) present in feedstuffs should be pursued to further improve recovery values.

## References

- AOAC, 2005. In: Horwitz, W., Latimer Jr., G.W. (Eds.), *Official methods of analysis*, 18th rev. ed. AOAC International, Gaithersburg, MD.
- Bach Knudsen, K.E., 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. *Anim. Feed Sci. Technol.* 67, 319–338.
- Browne, C.A., 1912. XXI. The trisaccharides and tetrasaccharides. In: *A Handbook of Sugar Analysis*. John Wiley & Sons, New York, pp. 731–750.
- Brummer, Y., Cui, S.W., 2005. Understanding carbohydrate analysis. In: Cui, S.W. (Ed.), *Food Carbohydrates: Chemistry, Physical Properties, and Applications*. Taylor & Francis Group, LLC, Boca Raton, FL, pp. 68–104 (Chapter 2).
- Buyse, J., Merckx, R., 1993. An improved colorimetric method to quantify sugar content of plant tissue. *J. Exp. Bot.* 44, 1627–1629.
- DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350–356.
- Hall, M.B., Hoover, W.H., Jennings, J.P., Miller Webster, T.K., 1999. A method for partitioning neutral detergent-soluble carbohydrates. *J. Sci. Food Agric.* 79, 2079–2086.
- Harding, V.J., Nicholson, T.F., Archibald, R.M., 1936. Some properties of the reducing material in certain fractions of normal urines: the nature of the “free” fermentable sugars and the fermentable sugars produced on hydrolysis in “fasting” urines. *Biochem. J.* 30, 326–334.
- Lever, M., 1973. Colorimetric and fluorometric carbohydrate determination with *p*-hydroxybenzoic acid hydrazide. *Biochem. Med.* 7, 274–281.
- Marten, D.A., Frankenberger Jr., W.T., 1990. Short communication: quantification of soil saccharides by spectrophotometric methods. *Soil Biol. Biochem.* 22, 1173–1175.
- SAS, 2010. *The SAS System for Windows*, Release 9.3. SAS Institute, Inc., Cary, NC.
- Southgate, D.A.T., 1976. *Determination of Food Carbohydrates*. Applied Science Publishers Ltd., London.
- Weinbach, A.P., Calvin, D.B., 1935. The reducing powers of physiologically important carbohydrates. *Science* 81, 407–408.